Mechanisms of Neonatal Mucosal Antibody Protection[1]

Nicola L. Harris,[2]*† Iris Spoerri,* Jacqueline F. Schopfer,* Chiara Nembrini,* Patrick Merky,* Joanna Massacand,* Joseph F. Urban, Jr.,‡ Alain Lamarre,§ Kurt Burki,* Bernhard Odermatt,* Rolf M. Zinkernagel,* and Andrew J. Macpherson*¶

Following an abrupt transition at birth from the sterile uterus to an environment with abundant commensal and pathogenic microbes, neonatal mammals are protected by maternal Abs at mucosal surfaces. We show in mice that different Ab isotypes work in distinct ways to protect the neonatal mucosal surface. Secretory IgA acts to limit penetration of commensal intestinal bacteria through the neonatal intestinal epithelium: an apparently primitive process that does not require diversification of the primary natural Ab repertoire. In contrast, neonatal protection against the exclusively luminal parasite *Heligmosomoides polygyrus* required IgG from primed females. This immune IgG could either be delivered directly in milk or retrotransported via neonatal Fc receptor from the neonatal serum into the intestinal lumen to exert its protective effect. ***The Journal of Immunology,*** **2006, 177: 6256–6262.**

The neonate acquires Igs from two routes: IgG passed transplacentally before birth and IgM, IgA, and IgG delivered via the milk (1). Indeed, breastfeeding is acknowledged to have immense power to avoid unnecessary human infant mortality, especially from diarrheal disease (2, 3). In rodents, IgG is also passed from the milk into the serum via the neonatal Fc receptor (FcRn)[3] present in the duodenum (4). FcRn can also transport IgG back from the serum into the intestinal lumen, in both rodents and humans (5–9), although the functional consequences of this process for neonates are unknown.

Neonates are inoculated at birth with a commensal microbiota in the lower intestine and other body surfaces. These commensals successively increase in density, and within weeks the microbes in the lower intestine outnumber the host's own cells (10). These commensals compete with pathogens and stimulate mucosal and immune development, but they share many surface molecules with pathogens including potentially damaging immunostimulatory compounds. Milk contains secretory Ig (S-Ig)A and S-IgM Abs that bind enteric pathogens or their toxins, which are partly induced in the maternal intestinal lymphoid follicles. Milk IgA and IgM are not absorbed significantly in any species and act locally in the intestinal lumen (11). Whether these secretory Abs need to undergo germinal center selection and somatic hypermutation for a neutralizing function against commensals or pathogens, or whether natural germline specificities suffice to provide a primitive exclusion mechanism has not been shown.

Although plasma IgG can contribute to protection from mucosal infection, it is present at lower concentrations in the milk of humans (12), and studies have mainly focused on uptake of milk IgG into the neonatal plasma as a means of transferring systemic protective immunity from mother to offspring. In humans and mice, IgG is transferred in utero to the infant bloodstream across the hemochorial placenta: after birth maternal IgG transfer continues until day 12–15 in mice, but not significantly in humans, via uptake of milk IgG through the FcRn in the intestinal epithelium (13, 14). In ungulates, maternal IgG is transferred from the colostrum via the intestinal FcRn in the first 12–18 h after birth (4). Despite these differences in systemic IgG uptake, all mammals are either born with high concentrations of plasma IgG (man), acquire it immediately after birth (ungulates), or contain significant IgG in milk (mouse). It is also appreciated that FcRn can transport IgG back from plasma into the intestinal lumen in mouse and man (5–7), and that neutralizing plasma IgG can protect neonates from infections entering through mucosal sites (15, 16). However, it is not known whether FcRn is critical for the protective function of IgG against infections by resecreting plasma Ab (previously absorbed from the mother) back onto the mucosal surface.

Materials and Methods

Animals and milk collection

C57BL/6 (B6), $J_H^{-/-}$, $IgA^{-/-}$, $RAG^{-/-}$, or β_2-microglobulin (β_2m)$^{-/-}$ mice were bred at the Labortierkunde of the University of Zürich under specific pathogen-free (SPF) conditions. All strains were maintained on the B6 genetic background. Germfree B6 and $J_H^{-/-}$ mice were maintained under gnotobiotic conditions at the Labortierkunde of the University of Zürich. Animal experiments were performed according to institutional guidelines and to Swiss federal and cantonal laws on animal protection. Milk was collected from lactating mice between days 14 and 16 postpartum. Pups were removed at least 3 h before the procedure, and females were given 1 IU of oxytocin (Syntocinon; Novartis) immediately before milking. Milking was performed under anesthesia using multiple vacuum-driven collection tubes attached to a flexible suction cap that was fitted over the mammary gland.

Bacterial challenge and counts

For enumeration of total culturable intestinal bacteria, serial dilutions of cecal contents were plated on LB agar for detection of aerobic bacteria, or Schaedler blood agar using an anaerobic hood (MG500; DW Scientific)

*Institute of Experimental Immunology, Universitätsspital, Zürich, Switzerland; †Environmental Biomedicine, Institute of Integrative Biology, Swiss Federal Institute of Technology, Zürich-Schlieren, Switzerland; ‡Nutrient Requirements and Functions Laboratory, Beltsville Human Nutrition Research Center, U.S. Department of Agriculture, Beltsville, MD 20705; §Institut National de la Recherche Scientifique-Institut Armand-Frappier, University of Quebec, Laval, Quebec, Canada; and ¶Department of Medicine, McMaster University Medical Centre, Hamilton, Ontario, Canada

Received for publication May 12, 2006. Accepted for publication August 3, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] A.J.M. was supported by the Swiss National Science Foundation and Canadian Institutes of Health Research. N.L.H. was supported by a Cancer Research Postdoctoral Fellowship.

[2] Address correspondence and reprint requests to Dr. Nicola L. Harris, Institute of Integrative Biology, Swiss Federal Institute of Technology, Wagistrasse 27, CH-8952 Zürich-Schlieren, Switzerland. E-mail address: nharris@ethz.ch

[3] Abbreviations used in this paper: FcRn, neonatal Fc receptor; S-Ig, secretory Ig; β_2m, β_2-microglobulin; SPF, specific pathogen-free; QM, quasimonoclonal.

0022-1767/06/$02.00

gassed with 85% N_2, 10% H_2, and 5% CO_2 for detection of anaerobic bacteria. Total bacterial counts were obtained by direct bacterial staining of smears using Accustain (Sigma-Aldrich). *Enterobacter cloacae* was grown by batch culture in LB medium and orally delivered at the indicated dose to postnatal day 18 pups. Translocation of *E. cloacae* across the intestinal epithelium was measured by plating serial dilutions of mesenteric lymph node cells onto LB agar.

Intestinal parasite infection

Female mice infected with *Heligmosomoides polygyrus* were inoculated orally with 200 larvae, treated with 250 μg of the antihelminth pyrantelum (Cobantril; Pfizer) 1 mo later, then subjected to a second infection with the same dose of larvae and breed 1 wk later. Pups were infected with 100–300 *H. polygyrus* larvae by oral gavage using thin-walled flexible tubing, and the number of adult parasites was determined between days 13 and 15 postinfection by examining whole intestine plus luminal contents under a dissecting microscope.

IgH chain sequence analysis

Analysis of V(D)J C cDNA sequences was conducted with oligo(dT)-primed cDNA that had been synthesized using mRNA from 0.1–0.5 g of ileum with the Peyer's patches removed. Sequences were amplified by PCR as described previously (17). Primers were 5′-CGGTGGTTATATC CTTCC-3′ (IgHα) and a degenerate (HB1-HB19) mix for the V_H region (17). PCR products were purified and subcloned into pGEM for automated sequencing.

Immunohistochemistry and Ab detection

Immunohistochemistry was performed as described previously (18). Serum titers of IgA were determined by ELISA, and Ab-secreting cells were quantified by ELISPOT as described previously (18). Milk parasite-specific Abs were measured by incubation of serially diluted samples onto 96-well plates coated with 5 μg/ml HES (excretory/secretary products collected from adult *H. polygyrus* cultured for a period of 2 days in RPMI 1640 plus antibiotics and 1% glucose, followed by concentration to $\geq$0.5 mg/ml by centrifugation through a 10,000 MWCO cellulose membrane, Centriprep; Millipore). Bound Ab was detected with secondary peroxidase-conjugated Abs to mouse IgG1 (Zymed) or IgA (Sigma-Aldrich) added at a dilution of 1/1000 in PBS and developed as described (18). To calculate Ab concentrations, an internal standard consisting of serum from mice infected two times with *H. polygyrus* larvae was used. Murine IgG was purified from serum by affinity chromatography (Protein G-Sepharose 4 Fast Flow; Amersham Pharmacia Biosciences).

Results

Milk Abs prevent neonatal responsiveness against commensal microbiota

Cebra and colleagues (19) showed that early neonatal mucosal immune induction occurred in pups nursed by scid females that were globally deficient in adaptive immunity, so we started by confirming that early stimulation of mucosal immunity occurred in pups nursed by milk from females with selective Ab deficiency. For this, we used $J_H^{-/-}$ mice that have no mature B cells and express no Ig of any isotype, because of deletion of the J segments in the IgH chain locus (20). We crossed $J_H^{-/-}$ mice (on a B6 background) with wild-type B6 partners. If the mother was $J_H^{-/-}$ and the father B6, the heterozygous $J_H^{+/-}$ offspring were immunocompetent because they carried a normal IgH chain allele, but they were nursed with Ab-deficient milk (Fig. 1*A*). Conversely, if the mother was B6 wild type, and the father $J_H^{-/-}$, the offspring had the same $J_H^{+/-}$ genotype, but in this case they were nursed by females with milk containing Abs (Fig. 1*B*). The offspring of B6 $\times$ B6 crosses were also studied as controls. This experimental design confined the differences to Ig transfer, rather than innate factors or T lymphocytes present in milk.

There was no nutritional disadvantage in the pups of these different crosses because weight gain was consistently identical between these groups (Fig. 1*B*), nor was there any difference in the densities of luminal intestinal bacteria colonizing the pups (Fig. 1*C*). However, despite equivalent intestinal bacterial loads, $J_H^{+/-}$ pups nursed by a $J_H^{-/-}$ female had early induction of plasma IgA and early appearance of IgA-producing cells in the intestinal mucosa by postnatal day 16 (Fig. 2, *A*, *B*, and *D*). Pups nursed by B6 females with milk containing Igs did not produce plasma IgA until after weaning on day 21 (Fig. 2*A*), and no IgA-producing intestinal plasma cells could be detected at either day 16 or day 20 of age (Fig. 2, *C* and *E*). Because exposure of the mucosal associated lymphoid tissues to commensal bacteria is known to drive mucosal IgA production, these data implied that pups nursed on Ab-deficient females were subject to greater commensal penetration. To demonstrate that exposure to commensal microbiota was responsible for the early IgA induction in the

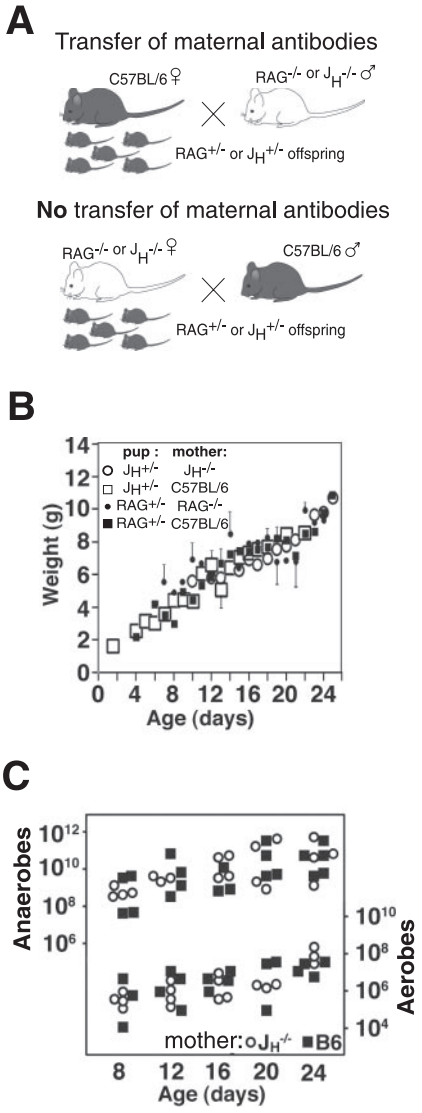

FIGURE 1. Absence of maternal milk Igs does not alter pups growth or intestinal commensal bacteria densities. *A*, Scheme showing breeding experiments whereby immunocompetent pups ($RAG^{+/-}$ or $J_H^{+/-}$) are nursed by a female in which Abs are present in (C57BL/6), or absent ($RAG^{-/-}$ or $J_H^{-/-}$), from the milk. *B*, Serial weights of $J_H^{+/-}$ offspring of $J_H^{-/-}$ (♀) $\times$ B6 (♂) or $J_H^{-/-}$ (♂) $\times$ B6 (♀) breedings, and $RAG^{+/-}$ offspring of $RAG^{-/-}$ (♀) $\times$ B6 (♂) or $RAG^{-/-}$ (♂) $\times$ B6 (♀) breedings. Values are mean $\pm$ SD ($n \geq 8$ pups), and error bars usually do not exceed the size of the symbols. B6 pups nursed by a B6 female had weights that were not significantly different from any of the above groups (data not shown). *C*, Caecal densities of aerobic or anaerobic bacteria in $J_H^{+/-}$ offspring of $J_H^{-/-}$ (♀) $\times$ B6 (♂) breedings. Direct bacterial staining showed no difference in density between $J_H^{+/-}$ pups nursed by $J_H^{-/-}$ (♀) (day 16 = $4.3 \pm 2.9 \times 10^{10}$; $n = 11$) or B6 (♀) (day 16 = $5.7 \pm 4.1 \times 10^{10}$; $n = 13$)

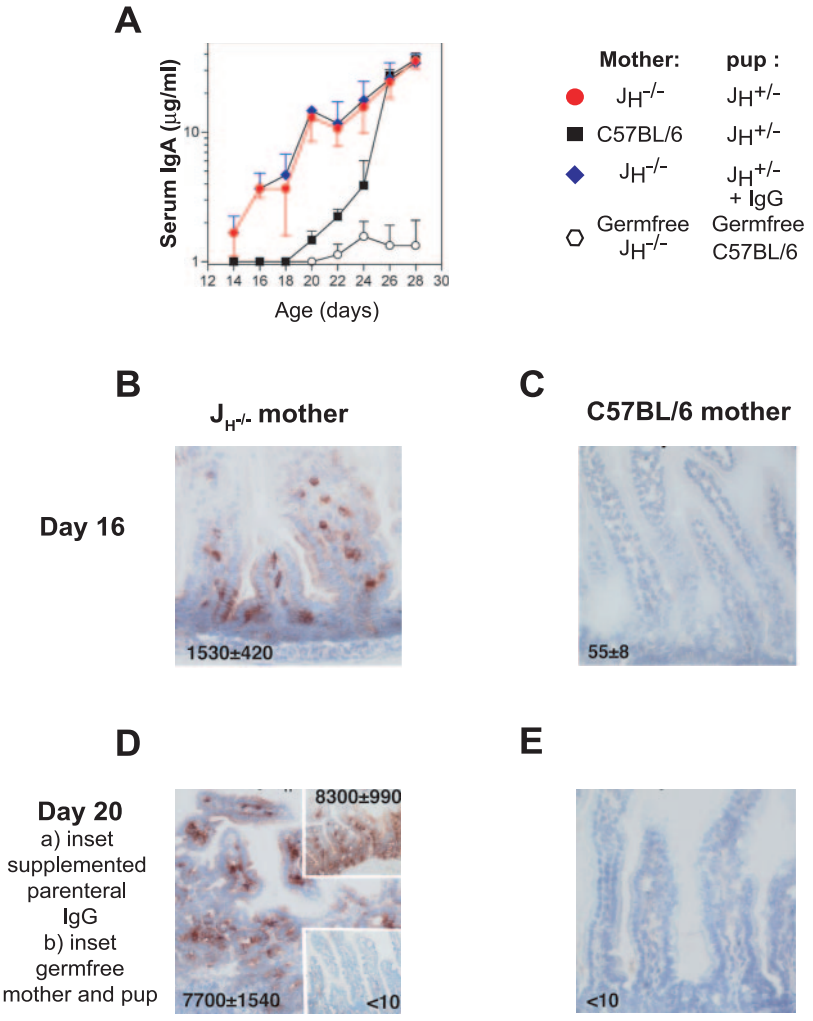

FIGURE 2. Premature activation of neonatal mucosal immunity in response to luminal commensal bacteria in the absence of maternal milk Igs. *A*, Serum IgA ($\bar{x}$ ± SD; $n \geq 3$) taken on different postnatal days for the following: $J_H^{+/-}$ pups nursed by $J_H^{-/-}$ (♀) (closed circles), $J_H^{+/-}$ pups nursed by B6 (♀) (■), $J_H^{+/-}$ pups nursed by $J_H^{-/-}$ (♀) and supplemented with parenteral purified SPF IgG (◆), or germfree B6 pups nursed by germfree $J_H^{-/-}$ (♀) (○). *B–E*, Sections of terminal ileum were stained for IgA-producing plasma cells in $J_H^{+/-}$ pups nursed by $J_H^{-/-}$ (♀) (*B* or *D*) or B6 (♀) (*C* or *E*). Analysis was on postnatal day 16 (*B* or *C*) or postnatal day 20 (*D* or *E*). Some $J_H^{+/-}$ pups nursed by $J_H^{-/-}$ (♀) were supplemented parenterally with purified IgG between days 6 and 18 (*D*, *top right inset*; the parenteral dose was matched to reflect wild-type concentrations: IgG in serum of day 12 supplemented pups = 198 ± 38 mg/ml; IgG in B6 progeny of B6 × B6 breedings = 173 ± 21 mg/ml). Terminal ileum at postnatal day 20 of germfree $J_H^{+/-}$ pup nursed by a germfree $J_H^{-/-}$ (♀) is shown (*D*, *bottom right inset*). Numbers of IgA-secreting cells/10^5 intestinal leukocytes, as determined by ELISPOT, are shown on each panel (mean ± SD; $n = 4$).

neonates, we conducted the $J_H^{-/-}$ (♂) × B6 (♀) breedings under entirely germfree conditions. The absence of intestinal bacteria resulted in a loss of the early mucosal and plasma IgA induction in the $J_H^{+/-}$ pups (Fig. 2*D*, *bottom right inset*), even though the female was not secreting Abs. There was also no difference in the kinetics of low levels of IgA induction between pups nursed by a $J_H^{-/-}$ or B6 female under germfree conditions (data not shown).

We next asked whether deficient IgG uptake with resulting plasma hypogammaglobulinaemia, or absence of secretory Abs from the milk, was the cause of early mucosal immune activation in the $J_H^{+/-}$ pups nursed by a $J_H^{-/-}$ female. Deficient transplacental IgG was not responsible for the effect because there was early induction of endogenous IgA at postnatal day 16 in pups born to B6 females and exchanged onto lactating $J_H^{-/-}$ females within 24 h of birth (Table I). Similarly, we supplemented some $J_H^{+/-}$ pups nursed by a $J_H^{-/-}$ female with daily s.c. injections with IgG purified from naive B6 serum to match serum concentrations to those pups receiving IgG from the milk, and found that this did not prevent early IgA induction (Fig. 2*D*, *top right inset*). These data formally show that secretory milk Abs, acting locally in the neonatal intestine, are responsible for protection against early mucosal immune activation. However, because accelerated induction of mucosal IgA production was not seen when B6 pups were exchanged onto $IgA^{-/-}$ females (Table I), it is likely that both S-IgA and IgM in the milk can protect the neonate from early mucosal immune activation.

Table I. *Induction of mucosal immune responses in pups suckling on mothers deficient in maternal Igs*

Mother[a]	Pup	Nurse[b]	Day 16[c] IgA-secreting cells/10^5 intestinal leukocytes	Day 20[c] IgA-secreting cells/10^5 intestinal leukocytes
$J_H^{-/-}$	$J_H^{+/-}$	B6	<10	56 ± 25
B6	B6	$IgA^{-/-}$	<10	33 ± 18
B6	$J_H^{+/-}$	$J_H^{-/-}$	1240 ± 680	5210 ± 3200
B6	B6	Not litterswapped	<10	<10

[a] Timed breedings were set up with harem mating of the strains shown.
[b] Pups were litterswapped to be nursed by a single female of the strain shown on day 1 after birth.
[c] IgA-secreting cells present in the small intestine of pups were measured by ELISPOT after extraction of intestinal leukocytes as previously described. Values are $\bar{x}$ ± SD, $n = 3$ or 4 pups, with results pooled from two to five litters.

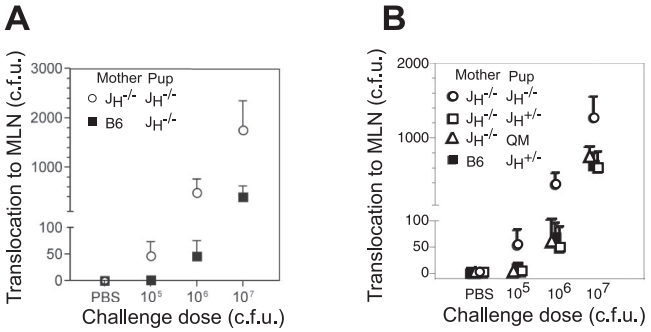

FIGURE 3. Undiversified Abs limit commensal penetration across the neonatal intestinal mucosa. *A*, Total bacterial counts from the mesenteric lymph node of $J_H^{-/-}$ pups nursed by $J_H^{-/-}$ (♀) (○) or B6 (♀) (■) were determined 16 h after intragastric challenge with indicated doses of *E. cloacae*. *B*, Total bacterial counts from the mesenteric lymph node of the following: $J_H^{+/-}$ pups nursed by $J_H^{-/-}$ (♀) (□), $J_H^{+/-}$ pups nursed by B6 (♀) (■), $J_H^{-/-}$ pups nursed by $J_H^{-/-}$ (♀) (○), or QM pups nursed by $J_H^{-/-}$ (♀) (△). Bacterial counts were determined 18 h after intragastric challenge with indicated doses of *E. cloacae*.

To more directly address whether maternal secretory Abs could prevent commensal translocation across the neonatal intestinal epithelium, we challenged pups with experimental doses ($0–10^7$ CFU) of *E. cloacae*, the dominant aerobe in our colony of SPF mice. At postnatal day 18, the presence of Abs in the milk of the lactating female protected pups from translocation of the different bacterial doses to the mesenteric lymph nodes, indicating functional exclusion of challenge doses of commensal organisms by maternal Abs (Fig. 3*A*).

Function of undiversified neonatal IgA in preventing commensal bacterial penetration

We additionally considered that early induction of IgA production in pups not receiving maternal Abs may serve to limit commensal penetration. In contrast to the Ab-deficient $J_H^{-/-}$ pups, immunocompetent $J_H^{+/-}$ pups lactated by a $J_H^{-/-}$ female were able to exclude challenge doses of commensal organisms, demonstrating the protective effect of early endogenous IgA production (Fig. 3*B*). The effectiveness of S-IgA produced by young pups suggested that "natural" germline IgA was functional for commensal exclusion. We therefore examined this protective effect in the setting of mAb secretion. For this experiment, we exchanged litters of quasimonoclonal (QM) pups, which have a targeted anti-nitrophenyl Ab specificity capable of class switching to all isotypes (21), onto Ab-deficient $J_H^{-/-}$ females for lactation. Just as for the $J_H^{+/-}$ pups, lactation by an Ab-deficient female resulted in early induction of mucosal IgA in the QM neonates (data not shown).

We additionally showed that endogenous mucosal Ab production in these circumstances was almost exclusively of the IgA isotype, and stage sequence analysis of the Igα H chain showed that the Ab produced in the pups was exclusively of the anti-nitrophenyl specificity (Table II), unlike in older adult mice where extensive receptor editing can occur (Table II) (22). Even this mAb repertoire present in day 18 QM pups nursed by $J_H^{-/-}$ females was effective in excluding challenge doses of *E. cloacae* (Fig. 3*B*), confirming that a natural IgA repertoire is sufficient for the exclusion function.

Table II. *Sequence analysis of lamina propria IgacDNA clones from day 18, day 25 and day 180 QM mice*

			Rearranged Sequence			Targeted D-J Segment Sequence				
Clone[a]	V_H	V_H Sequence	*n* Insertion	D Segment	*n* Insertion	DSP2.10	*n*	J_{H4}	Inserted D Segment Identity	Mice Analyzed
Day 18										
17.2.25	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		QM pups nursed
A3.1	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		by $J_H^{-/-}$ dam
A3.2	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
A3.3	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
A3.4	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
A3.5	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
A3.6	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
A3.7	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
A3.8	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
A3.9	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
A3.10	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
A3.11	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
A3.12	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
Day 25										
5.1	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		QM pups nursed
5.2	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		by QM dams
5.3	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
5.4	3	TACTGTGCTAGA	CCGGGG	TACGGTAGTAGC	CCA	TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
5.5	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
5.6	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
5.7	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
A3.8	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
A3.9	14	TACTGTGCTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
Day 180										
8.3	2	TACTGTGCCAGA	GATGGAAT	CTATGGT	AT	CTATAGGTAC	CCT	TACTATGCTATGGACTAC	DSP2.3	QM pups nursed
8.2	1	TTCTGCGTAAGA	ACCG			ACTATAGGTAC	CCT	TACTATGCTATGGACTAC	?	by QM dams
8.3	5	TACTGTGCACGC	GGGG	GATTACGAC	GAATA	TACTATAAGTAC	CCT	TACTATGCTATGGACTAC	DSP2.2	
8.4	2	TACTGTGCCAGA	GAAGGTA	ATGGTAACTAC	GGAA	ACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
8.5	8	TACTGTGTTCGA	ATGAT	CTATGTTAATTA	TCTTGTTAGA	TACTATAAATTG	CCT	TACTATGCTATGGACTAC		
8.6	14	TACTGTGGAAGA				TACTATAGATAC	CCT	TACTATGCTATGGACTAC		
8.7	3	TACTGTGCAAAA	TATCTCC	GGGAC	GGGG	ACTATAGGTAC	CCT	TACTATGCTATGGACTAC	DQ52	
8.8	3	TACTGTGTGAG		GGA	T	TACAGTAACTAC	GGA	GGACATATTATGGACTAC	?	
8.9	1	TTCTGTGCAAGA	GAGGA	TATGATTACG	ACGGGG	TACTATAGGTAC	CCT	TACTCTGCTATGGACTAC	DSP2.2	
8.10	14	TACTGTGGTAGA				TACTATAGGTAC	CCT	TACTATGCTATGGACTAC		
8.11	14	TACTGTGCTAGA	TACC	TCCCA	AAT	TCATTACTGCGC	TCT	TACTATGCTATGGACTAC	DQ52-C	

[a] RT PCR amplification of the IgαH chain was carried out from mRNA prepared from ileal biopsies of QM mice of the different ages shown in the Table. The cDNA was subcloned into pGEM and analyzed by nucleotide sequencing. In each case, an in-frame VDJα sequence was obtained. Diversification of the CDR3 region was found in day 180 mice by secondary rearrangement, but the day 18 and day 25 sequences were undiversified and corresponded to the original VDJ insertion sequence.

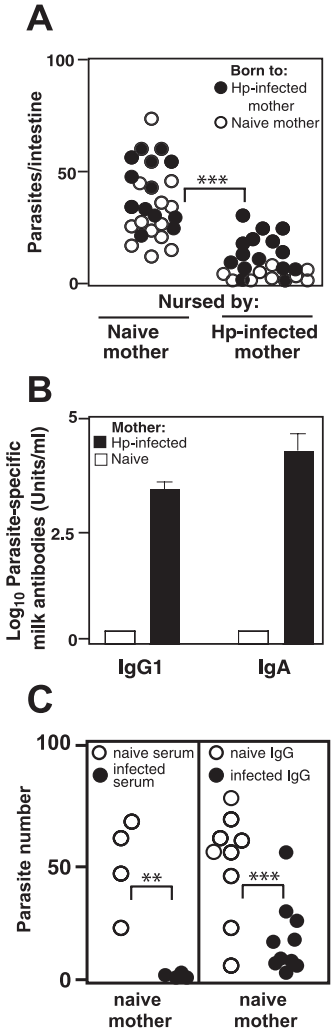

FIGURE 4. Immune maternal Abs protect neonates against enteric parasite infection. *A*, Day 10 B6 litters born to naive or *H. polygyrus* (Hp)-infected B6 (♀) mice were exchanged on the day of birth as indicated and infected with 100 Hp larvae. Numbers of adult parasites present in the intestines of pups were determined 13–15 days later. The significance between all pups nursed by naive or immune females was determined by a one-tailed Student's *t* test to be $p < 0.0001$. *B*, Titers of Hp-specific Abs present in the milk of naive or Hp-infected B6 (♀) mice are shown as Units/ml (mean ± SD; $n \geq 3$). Corresponding serum Ab titers in immune nursing females were 37,000 ± 48,073 IgG1 and 18,148 ± 15,012 IgA. *C*, B6 pups born to a naive female were supplemented parenterally between postnatal day 3 and 16 with serum or purified IgG from naive or Hp-infected adult B6 mice. Pups were infected with 100 Hp larvae at postnatal day 5, and parasite numbers were determined 13 days later. The significance between pups injected with naive or immune Abs was determined by a one-tailed Student's *t* test. A significant difference was noted for pups supplemented with naive or immune serum ($p = 0.002$), and for pups supplemented with purified naive or immune IgG ($p = 0.001$).

Prevention of mortality from neonatal parasite infection by maternal immune Abs

Whereas we showed above that natural Abs are sufficient to protect neonates against exposure to commensal intestinal bacteria, we found that nursing-dependent protection from neonatal intestinal parasite infection using *H. polygyrus*—a natural helminth parasite of mice with a strictly enteric lifecycle—requires prior priming of the females to be effective. We demonstrated that protective immunity to strictly local intestinal parasites can be passed from mother to neonate via the milk by the exchange of litters born to naive females onto immune females. This was most effective when the nursing females had been primed with two doses of *H. polygyrus* given between days 40–50, and again at day 7, before mating (Fig. 4*A*). Milk from these immune females contained high titers of parasite-specific IgG1 and IgA (Fig. 4*B*), and the peritoneal transfer of immune serum confirmed a role for these Abs in providing protection (Fig. 4*C*). Transfer of purified IgG also provided protection, indicating an important role for this isotype (Fig. 4*C*).

Importantly, the transfer of protective immunity from mother to offspring was demonstrated to be crucial for the survival and growth of offspring exposed to enteric parasites. Pups nursed by a naive female demonstrated stunted growth and morbidity when exposed to an increased number of parasites, necessitating their removal from the experiment, whereas pups nursed by an immune female remained healthy (Fig. 5).

FcRn transport of serum IgG into the intestinal lumen limits enteric parasite infection

To investigate whether the protective Abs functioned within the intestinal lumen, litters of β_2m-deficient pups, which lack expression of the FcRn responsible for transport of maternal IgG into the plasma (13), were exchanged onto immune B6 females. Wild-type B6 and $\beta_2 m^{-/-}$ pups were protected equally well by milk from immune females (Fig. 6*A*), despite an absence of immune IgG in the plasma of the $\beta_2 m^{-/-}$ pups (Fig. 6*B*). However, the maternal Abs did not need to be provided via the milk, because peritoneal transfer of total serum from immune mice was able to protect B6 pups, but not β_2m-deficient pups (Fig. 6*C*). This indicates that IgG can be retrotransported from the plasma into the intestinal lumen via a FcRn-mediated pathway (5), where it represents an important source of luminal protective Ab during the neonatal period.

Discussion

The immune integration between mother and neonate in mammals depends on transmission of maternal Igs via the placenta and/or through the milk (23). This allows the mother to transmit her immune experience that protects the offspring from environmental organisms and pathogens at a time when the neonatal immune system is immature. The fact that this process is extraordinarily effective has been well established. It is essential for newborn

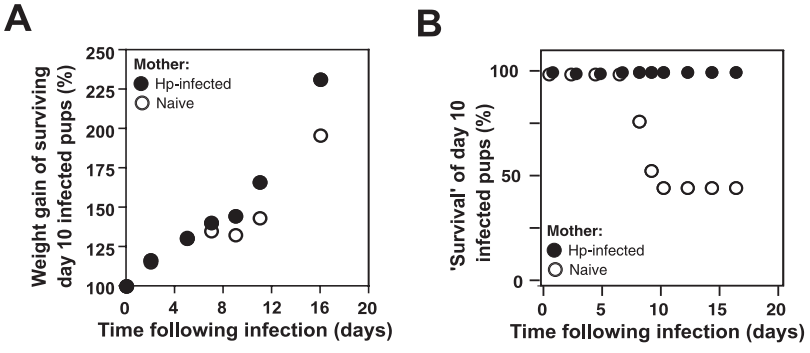

FIGURE 5. Immune maternal Abs prevent neonatal mortality following enteric parasite infection. Immune maternal Abs are necessary to ensure survival of neonates infected with enteric parasites. Pups nursed on naive ($n = 9$) or Hp-infected ($n = 13$) B6 (♀) were infected with 300 Hp larvae at day 10 of age then monitored twice daily. *A*, Serial weights (mean ± SEM, error bars do not exceed the size of the symbols) or (*B*) percentage of survival (mean) for all infected pups are shown from the time of infection.

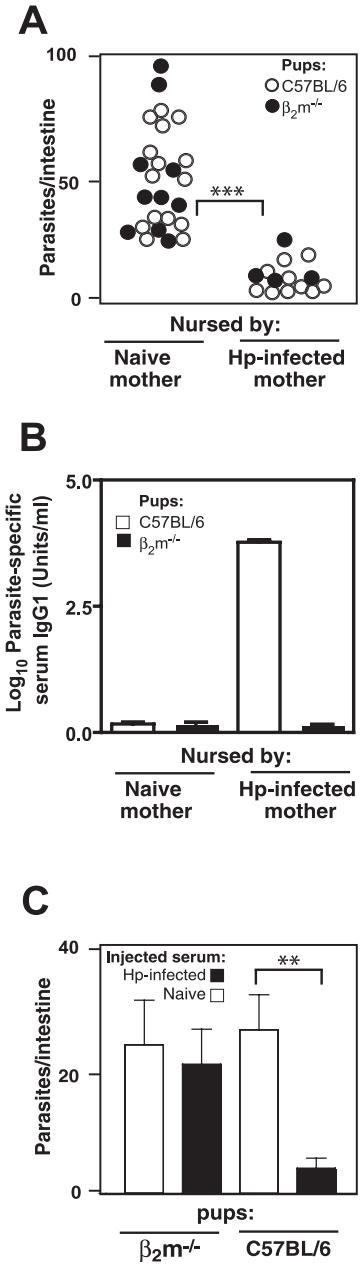

FIGURE 6. Immune maternal IgG must be present within the neonatal intestinal lumen to limit enteric parasite infection. *A*, Day 10 $\beta_2m^{-/-}$ or B6 pups were nursed on naive or Hp-infected B6 (♀) infected with 100 Hp larvae, and adult parasite numbers were determined 13 days later. The significance between all pups nursed by naive or immune females was determined by a one-tailed Student's *t* test to be $p < 0.0001$. *B*, Titers of Hp-specific IgG1 Abs present in the serum of $\beta_2m^{-/-}$ or B6 pups nursed on naive or Hp-infected B6 (♀) mice are shown as Units/ml (mean ± SEM; $n \geq 4$. *C*, $\beta_2m^{-/-}$ ($n = 13$) or B6 ($n = 16$) pups born to, and nursed by, a naive female were supplemented parenterally between postnatal day 3 and 16 with serum from naive or Hp-infected adult B6 mice. Pups were infected with 100 Hp larvae at postnatal day 5, and parasite numbers were determined 13 days later. Values represent mean ± SD. A significant difference, as determined by a one-tailed Student's *t* test, was noted for B6 pups ($p = 0.001$), but not $\beta_2m^{-/-}$ pups ($p = 0.367$), injected with naive or immune serum.

artiodactyls including foals, lambs, kids, and piglets to suckle colostrum if they are to survive, because in these species IgG is taken up through the intestine in the early postnatal period rather than across the placenta (4). In humans, where transplacental IgG transfer is essentially complete at birth, it is well established that breastfeeding offers protection from infectious disease especially at mucosal surfaces, and it has been calculated that increased use of exclusive breastfeeding would be the single most powerful method of preventing avoidable deaths worldwide (2). Although Ab neutralization of pathogens by S-IgA Abs that are delivered directly to mucosal surfaces has also been well established (reviewed in Ref. 24), in this study we have studied two additional aspects of neonatal mucosal Ab function in mice: first, the way in which secretory Abs of the IgA and IgM isotypes act to exclude intestinal commensal bacterial Ags; and second, the neutralizaton of mucosal pathogens by plasma IgG that has been transported into the intestinal lumen via the FcRn.

IgA can be induced both in a Th cell-dependent fashion, where high-affinity neutralizing Ab is generated (25), or by a more primitive cognate Th cell-independent pathway generating poly-reactive Abs with limited diversity (26–28). IgA is the predominant Ig in milk of both mice and humans, and in mice it has been shown that IgA plasma cells in the mammary gland derive from lymphoblast precursors in the gut-associated lymphoid tissue (29).

Rejnek et al. (11) first demonstrated that Abs delivered to the intestine of germfree piglets could act locally to protect against lethal peroral doses of pathogenic *Escherichia coli*. Kramer and Cebra (19) then showed that neonatal mucosal immune induction was delayed in immunocompetent pups nursed by *scid* females. They also conducted flow cytometry experiments to show that intestinal IgA produced by postnatal day 16 in scid/+ pups nursed by a scid/scid female bound to commensal intestinal bacteria. When pups were nursed by a wild-type mother, milk IgA could be seen to coat intestinal bacteria at days 10 and 13, before endogenous IgA is induced. The interpretation from this work that milk IgA protects the neonatal mucosa from early induction driven by commensal bacteria and their products had some caveats: first, maternal IgG transfer via the milk into the neonatal plasma and the content of milk lymphocytes would both have been deficient in pups nursed by *scid/scid* females; and second, early induction of the neonatal immune system or exclusion of commensal bacteria was not directly shown. Our data resolve these points in an experimental design, where immunocompetent pups are nursed by females that are selectively Ab deficient, and where plasma IgG levels have been corrected by parenteral supplementation. We have shown directly using germfree conditions that it is the commensal microbiota that drive the early induction of intestinal IgA-producing cells in colonized immunocompetent pups nursed by an Ab-deficient female (Fig. 2, *A* and *D*). Plasma IgA was first detected in these neonates at a similar time as the first appearance of intestinal IgA-secreting cells, and was likely derived from the intestinal IgA-producing cells (Fig. 2) (30). Finally, we show that the early monoclonal IgA produced in QM (nitrophenol specificity; Ref. 21) Ab knock-in pups nursed by Ab-deficient females is sufficient to exclude challenge doses of commensal bacteria: this result suggests that even undiversified natural IgA can perform commensal bacterial exclusion. Because young (<6 wk) QM mice have no measurable lamina propria plasma cells producing other Ab isotypes (data not shown), S-IgA is sufficient for the effect. Additional molecules with antimicrobial or exclusion effects, including free secretory component, are present in milk (23); however, S-IgA has a clear effect on immune exclusion and protection of the neonatal mucosa against commensal intestinal bacteria.

IgG is only present at very low concentrations in human milk, although antenatal uptake via the placenta allows an infant to acquire substantial amounts of protective maternal IgG in the plasma by the time it is born. In contrast, newborn artiodactyls absorb IgG from the colostrums via the intestine early after birth, and this

isotype remains dominant in milk, even after closure of the intestinal uptake pathway (4). Given the fact that the FcRn, which is responsible for duodenal IgG uptake from the milk in rodents (13), is expressed in human epithelial cells (8), and can shuttle IgG in the reverse direction from the serosal to the mucosal surface (5, 31), it is important to know whether this can be a mechanism for delivering protective IgG to the mucosal surface. Indeed parenteral or i.v. administration of neutralizing IgG has been shown to protect neonatal monkeys from SIV infection (15, 16), although the mechanisms were not defined. We modeled the effects of IgG at the mucosal surface in mice using the luminal helminth infection *H. polygyrus*, because helminth infections are extremely common in nonindustrialized human populations, and are thought to have severe consequences for childhood nutrition and development (32).

In contrast to the exclusion mechanism by undiversified IgA against commensal intestinal bacteria, protection of neonates against potentially lethal doses of *H. polygyrus* requires transfer of IgG during nursing by a previously primed female. Although protective IgG can be delivered directly in the milk, plasma IgG after parenteral administration is also effective. The mechanism of delivery of protective IgG from the plasma to the intestinal lumen against this helminth has been shown in vivo to depend on the FcRn, because targeted deletion of β_2m, a component of FcRn (13), abrogates the effect. Although IgG can be directly delivered in the milk of mice and other mammals, retrograde protective IgG transport from the plasma may be especially relevant in humans, where milk IgG concentrations are low but there has been transfer of maternal IgG across the placenta before birth to the neonatal plasma.

In summary, our experiments show the different mechanisms of Ab-mediated protection at the neonatal mucosal surface. Milk IgA and IgM transfers the ability from mother to neonates to provide exclusion of luminal bacteria, and this process is not critically dependent on Ab specificity. In contrast, parasite-specific IgG transfer from immune mothers is important to protect neonates against mortality resulting from enteric parasite infection with *H. polygyrus*. This IgG must be present within the intestinal lumen to be functional and can be delivered either through the milk or from the neonatal plasma by FcRn-mediated transport.

Acknowledgments

We thank Dr. Barbara Koenig for help with the collection of murine milk and Therese Uhr for technical support.

Disclosures

The authors have no financial conflict of interest.

References

1. Hanson, L. A., M. Korotkova, and E. Telemo. 2005. Human milk: its component and their immunobiologic functions. In *Mucosal Immunology.* J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, J. R. McGhee, and L. Mayer, eds. Elsevier, Amsterdam, pp. 1796–1827.
2. Jones, G., R. W. Steketee, R. E. Black, Z. A. Bhutta, and S. S. Morris. 2003. How many child deaths can we prevent this year? *Lancet* 362: 65–71.
3. Labbok, M. H., D. Clark, and A. S. Goldman. 2004. Breastfeeding: maintaining an irreplaceable immunological resource. *Nat. Rev. Immunol.* 4: 565–572.
4. Butler, J. E., and M. E. Kehrli. 2005. Immunoglobulins and immunocytes in the mammary gland and its secretions. In *Mucosal Immunology.* J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, J. R. McGhee, and L. Mayer, eds. Elsevier, Amsterdam, pp. 1763–1793.
5. Yoshida, M., S. M. Claypool, J. S. Wagner, E. Mizoguchi, A. Mizoguchi, D. C. Roopenian, W. I. Lencer, and R. S. Blumberg. 2004. Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. *Immunity* 20: 769–783.
6. Claypool, S. M., B. L. Dickinson, M. Yoshida, W. I. Lencer, and R. S. Blumberg. 2002. Functional reconstitution of human FcRn in Madin-Darby canine kidney cells requires co-expressed human β_2-microglobulin. *J. Biol. Chem.* 277: 28038–28050.
7. Dickinson, B. L., K. Badizadegan, Z. Wu, J. C. Ahouse, X. Zhu, N. E. Simister, R. S. Blumberg, and W. I. Lencer. 1999. Bidirectional FcRn-dependent IgG transport in a polarized human intestinal epithelial cell line. *J. Clin. Invest.* 104: 903–911.
8. Israel, E. J., S. Taylor, Z. Wu, E. Mizoguchi, R. S. Blumberg, A. Bhan, and N. E. Simister. 1997. Expression of the neonatal Fc receptor, FcRn, on human intestinal epithelial cells. *Immunology* 92: 69–74.
9. Ghetie, V., and E. S. Ward. 1997. FcRn: the MHC class I-related receptor that is more than an IgG transporter. *Immunol. Today* 18: 592–598.
10. Mackie, R., A. Sghir, and H. R. Gaskins. 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. *Am. J. Clin. Nutr.* 69: 1035S–1045S.
11. Rejnek, J., J. Travnicek, J. Kostka, J. Sterzl, and A. Lanc. 1968. Study of the effect of antibodies in the intestinal tract of germ-free baby pigs. *Folia Microbiol.* 13: 36–42.
12. Mehta, P. D., S. P. Mehta, and C. E. Isaacs. 1989. Distribution of IgG subclasses in human colostrum and milk. *Immunol. Lett.* 22: 235–238.
13. Jacobowitz Israel, E., V. K. Patel, S. F. Taylor, A. Marshak-Rothstein, and N. E. Simister. 1995. Requirement for a β_2-microglobulin-associated Fc receptor for acquisition of maternal IgG by fetal and neonatal mice. *J. Immunol.* 154: 6246–6251.
14. Simister, N. E., and K. E. Mostov. 1989. An Fc receptor structurally related to MHC class I antigens. *Nature* 337: 184–187.
15. Baba, T. W., V. Liska, R. Hofmann-Lehmann, J. Vlasak, W. Xu, S. Ayehunie, L. A. Cavacini, M. R. Posner, H. Katinger, G. Stiegler, et al. 2000. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nat. Med.* 6: 200–206.
16. Mascola, J. R., G. Stiegler, T. C. VanCott, H. Katinger, C. B. Carpenter, C. E. Hanson, H. Beary, D. Hayes, S. S. Frankel, D. L. Birx, and M. G. Lewis. 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat. Med.* 6: 207–210.
17. Krebber, A., S. Bornhauser, J. Burmester, A. Honegger, J. Willuda, H. R. Bosshard, and A. Pluckthun. 1997. Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a re-engineered phage display system. *J. Immunol. Methods* 201: 35–55.
18. Macpherson, A. J., A. Lamarre, K. McCoy, G. Dougan, G. Harriman, H. Hengartner, and R. Zinkernagel. 2001. IgA B cell and IgA antibody production in the absence of μ and δ heavy chain expression early in B cell ontongeny. *Nat. Immunol.* 2: 625–631.
19. Kramer, D. R., and J. J. Cebra. 1995. Early appearance of "natural" mucosal IgA responses and germinal centers in suckling mice developing in the absence of maternal antibodies. *J. Immunol.* 154: 2051–2062.
20. Chen, J. Z., M. Trounstine, F. W. Alt, F. Young, C. Kurahara, J. F. Loring, and D. Huszar. 1993. Immunoglobulin gene rearrangement in B-cell deficient mice generated by targeted deletion of the J_H locus. *Int. Immunol.* 5: 647–656.
21. Cascalho, M., A. Ma, S. Lee, L. Masat, and M. Wabl. 1996. A quasi-monoclonal mouse. *Science* 272: 1649–1652.
22. Cascalho, M., J. Wong, and M. Wabl. 1997. V_H gene replacement in hyperselected B cells of the quasimonoclonal mouse. *J. Immunol.* 159: 5795–5801.
23. Brandtzaeg, P. 2003. Mucosal immunity: integration between mother and the breast-fed infant. *Vaccine* 21: 3382–3388.
24. Hanson, L. A., M. Korotkova, and E. Telemo. 2005. Human milk: its components and their immunobiologic functions. In *Mucosal Immunology.* J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, J. R. McGhee, and L. Mayer, eds. Elsevier, Amsterdam, pp. 1795–1827.
25. Lycke, N., L. Eriksen, and J. Holmgren. 1987. Protection against cholera toxin after oral immunisation is thymus dependent and associated with intestinal production of neutralising IgA antitoxin. *Scand. J. Immunol.* 25: 413–419.
26. Quan, C. P., A. Berneman, R. Pires, S. Avrameas, and J. P. Bouvet. 1997. Natural polyreactive secretory immunoglobulin A autoantibodies as a possible barrier to infection in humans. *Infect. Immun.* 65: 3997–4004.
27. Macpherson, A. J., D. Gatto, E. Sainsbury, G. R. Harriman, H. Hengartner, and R. M. Zinkernagel. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 288: 2222–2226.
28. Stoel, M., H.-Q. Jiang, C. C. van Diemen, J. C. A. M. Bun, P. M. Dammers, M. C. Thurnheer, F. G. M. Kroese, J. J. Cebra, and N. A. Bos. 2005. Restricted IgA repertoire in both B-1 and B-2 cell-derived gut plasmablasts. *J. Immunol.* 174: 1046–1054.
29. Roux, M. E., M. McWilliams, J. M. Phillips-Quagliata, P. Weisz-Carrington, and M. E. Lamm. 1977. Origin of IgA-secreting plasma cells in the mammary gland. *J. Exp. Med.* 146: 1311–1322.
30. Peppard, J. V., C. S. Kaetzel, and M. W. Russell. 2005. Phylogeny and comparative physiology of IgA. In *Mucosal Immunology.* J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, J. R. McGhee, and L. Mayer, eds. Elsevier, Amsterdam, pp. 195–210.
31. McCarthy, K. M., Y. Yoong, and N. E. Simister. 2000. Bidirectional transcytosis of IgG by the rat neonatal Fc receptor expressed in a rat kidney cell line: a system to study protein transport across epithelia. *J. Cell Sci.* 113: 1277–1285 (Pt. 7).
32. WHO. 1997. WHO information series on school health. Document 1. Strengthening interventions to reduce helminth infections: an entry point for the development of health-promoting schools. WHO, Geneva.